Protection of *Aotus* Monkeys by *Plasmodium falciparum* EBA-175 Region II DNA Prime–Protein Boost Immunization Regimen

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Aotus monkeys received 4 doses of *Plasmodium falciparum* EBA-175 region II vaccine as plasmid DNA (Dv-Dv) or recombinant protein in adjuvant (Pv-Pv) or as 3 doses of DNA and 1 dose of protein (Dv-Pv). After 3 doses, antibody titers were ~10^4 in DNA-immunized monkeys and 10^6 in protein-immunized monkeys. A fourth dose did not significantly boost antibody responses in the Dv-Dv only or Pv-Pv only groups, but titers were boosted to ~10^6 in monkeys in the Dv-Pv group. Four weeks after the last immunization, the animals were challenged with 10^4 *P. falciparum*–parasitized erythrocytes. Peak levels of parasitemia were lower in the 16 monkeys that received region II–containing plasmids or proteins than in the 16 controls (geometric mean: 194,178 and 410,110 parasites/μL, respectively; $P = .013$, Student's t test). Three of 4 monkeys in the Dv-Pv group did not require treatment. These data demonstrate that immunization with EBA-175 region II induces a significant antiparasite effect in vivo.

We believe that effective malaria vaccines will contain a variety of immunogens selected to direct different immune responses to multiple antigens from several stages of the *Plasmodium* life cycle [1, 2]. Preventing parasite invasion of erythrocytes would stop parasite multiplication and all clinical manifestations of the infection. Even vaccines that only reduce, rather than eliminate, parasite burden may reduce malaria morbidity and mortality in vulnerable populations (e.g., children and pregnant women) in endemic areas.

EBA-175, the 175-kD *Plasmodium falciparum* erythrocyte-binding protein, is the merozoite ligand for glycophorin A, the primary known *P. falciparum* receptor on erythrocytes [3, 4]. EBA-175 contains a 616 amino acid, N-terminal cysteine-rich region [4] called region II [5], which contains the receptor-binding domain of EBA-175 [6]. The functional importance of region II, the fact that region II is highly conserved [7], and the fact that all *P. falciparum* strains studied to date express EBA-175 reinforce the importance of using region II as a receptor blockade vaccine.

DNA vaccines offer several advantages over conventional synthetic or recombinant vaccines and are particularly promising for developing multistage, multiantigen malaria vaccines [2]. However, difficulties in inducing protective antibody levels with DNA vaccination alone have led to approaches that use DNA vaccination to prime the immune system and recombinant protein [8–11] or recombinant virus [12–18] to boost the immune response. We already have shown that *Aotus* monkeys immunized with a DNA vaccine encoding EBA-175 had a 10-fold increase in specific antibody to EBA-175 region II after challenge, which provides evidence of boosting induced by native protein expressed by parasites [19].

In this study, we evaluated the immunogenicity and protective efficacy in *Aotus* monkeys of a DNA vaccine encoding region II of *P. falciparum* EBA-175, a purified recombinant region II vaccine in adjuvant (Montanide 750 with CpG oligodeoxynucleotide), and a DNA prime–recombinant protein boost approach.

Materials and Methods

Parasites. *P. falciparum* parasite strains 3D7 (human strain) and FVO (*Aotus* adapted) were maintained for in vitro studies, as

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Experiments reported here were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," published by the Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996.

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reported elsewhere [20]. A temperature-cycling incubator (Forma Scientific) was used for maintaining parasite synchrony for in vitro growth studies. When necessary, schizonts were purified on Percoll density gradients [21]. *P. falciparum* FVO strain was metabolically labeled with TRAN^{35}S-LABEL (ICN Radiochemicals), as described elsewhere, and labeled culture supernatants were stored at −70°C [22]. The *P. falciparum* FVO strain (gift from Louis H. Miller, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) was used for *Aotus* challenge studies.

EBA-175 region II vaccines (DNA and protein) and adjuvants. *P. falciparum* EBA-175 region II (amino acids 145–760) was amplified from FVO genomic DNA and cloned into the DNA vector VR1050 (Vical), which uses the human cytomegalovirus promoter and intron A, human tissue plasminogen activator secretory signal, and the bovine growth hormone transcriptional terminator–polyadenylation site. The gene fragment encoding the universal T-epitopes from tetanus toxin P2P30 [23] was inserted 5′ of the region II gene, and the resultant construct was designated VR2527. VR1050 without insert was used as a control. Vical performed all plasmid production and purification for nonhuman primate studies, as described elsewhere [24, 25]. The generation, characterization, and production of the recombinant baculovirus EBA-175 region II protein has been reported elsewhere [26]. VR1721, which contains the gene encoding *Aotus* granulocyte-monocyte colony-stimulating factor (GM-CSF), was coadministered with both VR2527 and an empty vector control. The GM-CSF sequence used in the synthesis of VR1721 was a gift from Francois Villinger (Emory University, Atlanta, GA). EBA-175 region II protein (100 μg) or PBS control and 500 μg of oligodeoxynucleotide (ODN) 1968, which contains 3 immunostimulatory CpG motifs that are underlined (TCGTCGCTGTTGTCGTTTCTT) [27], were emulsified in adjuvant (Montanide ISA 720; Seppic) at a ratio (vol/vol) of 3:7 (total volume equal to 1 mL). CpG ODN 1968 was provided by Coley Pharmaceutical Group and contained <0.01 EU/mg endotoxin by limulus amebocyte lysate assay. The preparation was emulsified by vortexing that continued until a 5-μL drop of the emulsion failed to disperse when dropped into ice water.

Animals and immunizations. Thirty-four *Aotus nancymae* monkeys (supplied by the Center for Reproduction and Conservation of Non-human Primates, Iquitos, Peru; 16 males and 18 females), ranging in age from 1.5 to 2.5 years, were used in the study. Male and female pairs were randomly divided into 8 groups of 4 monkeys, with 2 female monkeys being maintained as parasite donors. The groups were as follows: Group 1 (Dv-Dv) received 4 doses of EBA-175 DNA (VR2527) mixed with *Aotus* GM-CSF DNA (VR1721); group 2 (Dv-Pv) received 3 doses of the EBA-175–GM-CSF DNA (VR2527/VR1721) mixture, which was followed by a dose of recombinant protein in adjuvant; group 3 (Dv-DvPv) received 3 doses of the EBA-175–GM-CSF DNA (VR2527/VR1721) mixture, and a final immunization with both the VR2527/VR1721 mixture and recombinant protein in adjuvant; group 4 (Dc-Dc) received 4 doses of control DNA (VR1050) plus GM-CSF DNA (VR1721); group 5 (Dc-DcPc) received 3 doses of the control–GM-CSF (VR1050/VR1721) DNA mixture, which was followed by 1 dose of the VR1050/VR1721 mixture and 1 dose of adjuvant; group 6 (Pv-Pv) received 4 doses of recombinant protein in adjuvant; group 7 (Pc-Pc) received 4 doses of adjuvant; and group 8 (n-n) were naive monkeys.

Monkeys were immunized at weeks 0, 4, 8, and 24. For DNA immunizations, each animal received 500 μg VR2527 or VR1050 and 500 μg of VR1721 plasmid DNA by intradermal inoculation, using a 0.3-mL insulin syringe with a fused 29-gauge 0.5-inch needle (Becton-Dickinson). The maximal volume administered in any one site was 100 μL, with each animal receiving injections at an average of 6 sites. EBA-175 region II protein with CpG ODN or saline control with CpG ODN emulsified in adjuvant (Montanide ISA 720) were administered subcutaneously (sc) bilaterally in each animal's back (total volume, 1 mL) and intramuscularly (im) bilaterally into the quadriceps muscles (total volume, 1 mL), 1 site in each leg. Each animal received either 100 μg of the protein and 500 μg of the Montanide 720/ODN sc and im or PBS and 500 μg of the Montanide 720/ODN sc and im.

ELISA. Serum antibodies were assayed as described elsewhere [26]. Purified baculovirus recombinant EBA-175 region II protein (1 μg/mL) in PBS/sodium azide buffer was used as capture antigen. ELISA results are reported as the interpolated reciprocal dilution estimated to give an optical density (OD) of 0.5.

Indirect immunofluorescence technique (IFAT). IFAT slides were made as a single erythrocyte layer with a 5%–10% *P. falciparum* (FVO strain) parasitemia. The erythrocytic stages included mostly mature schizonts, but some late trophozoites and early schizonts were also present. Parasites were washed 3 times in PBS containing 2% bovine serum albumin, were dispensed in slide wells, and were left to air-dry before storing at −80°C. After the slides were thawed in a dehumidifier, duplicate wells were overlaid with 15 μL of the test and control antibody dilutions and were incubated in a moist chamber at 37°C for 60 min. Fluorescence detection was done with fluorescein isothiocyanate–labeled goat anti–human IgG (ICN Biomedical) diluted in PBS containing 0.05% Evans Blue. Slides were mounted with a cover slip, using mounting medium (Vectashield; Vector Laboratories), and were examined under an Olympus UV microscope. End-point titers were determined as the last dilution above the background that fluorescent parasites were observed. Background fluorescence was established for each group by using preimmunization serum samples. Positive control antibody was mouse polyclonal serum raised against a *P. falciparum* AMA-1 DNA vaccine.

Growth or invasion inhibition assay. For the analysis of antibody-mediated blockade, *Aotus* polyclonal IgG was purified from *Aotus* study groups 1–8 by column chromatography (Protein G; Pharmacia), using a buffer system (Immunopure; Pierce), as described elsewhere [28]. Parasite cultures were prepared as follows: 150-μL microcultures were prepared in triplicate, as for static cultures [20], but were kept suspended in 48-well plates on a rotator platform. In brief, synchronized cultures of *P. falciparum* schizont–infected erythrocytes at ~0.04% (FVO) or 0.02% (3D7) parasitemia were mixed with test or control IgG, so that final IgG concentrations were ~1 mg/mL, and the final hematocrit was 6%. A final 10% heat-inactivated normal human serum was included in all microcultures with bicarbonate-containing RPMI 1640 and was gassed with 5% O_2, 5% CO_2, and 90% nitrogen. Microculture plates were harvested for flow cytometry, as described elsewhere [28]. Percentage of inhibition (I) was calculated from the mean parasitemias of triplicate test and control wells as

100 × (control − test)/control. Additional calculations of inhibition per cycle (I_{pc}) were made by the equation $I_{pc} = 1 - (1 - I)^{1/2}$.

Blocking of native EBA-175 binding to erythrocytes. [^{35}S]-metabolically labeled parasite culture supernatant containing labeled EBA-175 was used for binding assays, as described elsewhere [6]. Antisera were diluted in RPMI 1640 with 10% fetal calf serum for the blocking assay. One-to-ten dilutions were prepared, and the samples bracketing an IC_5 were used to estimate the serum dilution that would produce a 50% inhibition. For a control, we used the counts from EBA-175 bound to erythrocytes in the presence of normal *Aotus* serum samples.

Challenge. On day 0, 1 mL of blood was obtained from a donor monkey with a *P. falciparum* parasitemia of 290 parasites/μL blood in ACD anticoagulant (Red Cross). The blood was diluted with 37°C RPMI 1640, so that 1 mL contained 10^4 parasitized erythrocytes. Two of 4 monkeys from each group were inoculated intravenously with 1 mL of the parasite preparation in the following order: group 1, 2, 3, 4, 5, 6, 7, 8. The last of the 16 monkeys was inoculated 62 minutes after the blood was drawn from the donor monkey. A blood sample was again obtained from the donor monkey, and the second 16 monkeys (2 from each group) were inoculated in the following order: group 7, 6, 5, 4, 3, 2, 1, 8.

Follow-up. Animals were monitored daily, starting on day 2, for parasites. The daily blood films (thick films only) were prepared from blood taken from the saphenous vein. The films were air-dried and were stained with Giemsa stain. Parasitemias were evaluated by the Earle-Perez method [29]. Ten microliters of blood was dispensed onto a measured area on a microscope slide. Parasitemias were calculated as parasites per microliter of blood. Hematocrit levels were determined daily. Serum samples were collected 2 and 4 weeks after challenge. A monkey was considered to require treatment if parasitized erythrocytes increased to ≥300,000 parasites/μL (7.5%) or if the hematocrit decreased by >50% over preinfection values. Infected monkeys were treated with 1 oral dose of mefloquine (20 mg/kg). Protection was defined as the ability on the part of the monkey to self-control its *P. falciparum* infection (i.e., protected monkeys did not require drug treatment for parasitemia or anemia during the 28-day follow-up). Monkeys that did not require treatment during follow-up were treated on day 28 with mefloquine. After treatment, the monkeys were monitored twice weekly until there was no sign of parasites in Giemsa-stained thick films, and the hematocrit levels had returned to normal.

Results

Antibody responses. Antibody titers in serum samples obtained immediately before and 4 weeks after each immunization were measured by ELISA. Means of $\log_{10}$-transformed titers for groups 1 (Dv-Dv), 2 (Dv-Pv), 3 (Dv-DvPv), and 6 (Pv-Pv) were compared. Four weeks after the third immunization, the mean for group 6 was higher than for the other 3 groups, as determined by 1-way analysis of variance (ANOVA) with Tukey honestly significant difference (HSD) post hoc test ($P < .05$). Four weeks after the fourth immunization (just before challenge), the means for groups 2 (Dv-Pv), 3 (Dv-DvPv), and 6 (Pv-Pv) were homogeneous and were all higher than the mean of group 1 (Dv-Dv; $P < .005$, by 1-way ANOVA with Tukey HSD post hoc test).

Groups 1, 2, and 3 all received the same immunogen formulation (DNA vaccine encoding *P. falciparum* EBA-175 region II) for the first 3 immunizations. To improve our power to analyze the immunogenicity of this DNA vaccine alone, we pooled the data from groups 1–3 ($n = 12$) for the first 3 immunizations. Four weeks after the first, second, and third immunizations, the mean ELISA titers for groups 1–3 were 99, 3690, and 13,959 respectively. Each mean was statistically different from the other 2 (1 vs. 2, $P < .0009$; 1 vs. 3, $P < .0009$; and 2 vs. 3, $P = .005$; by 1-way ANOVA with Tukey's HSD post hoc test), indicating that each additional immunization improved antibody responses. In group 1 ($n = 4$), the antibody titer after the fourth dose was 23,232, compared with 11,483 after the third dose ($P = .42$, Student's t test).

A similar evaluation was done to determine whether subsequent protein immunizations after the first immunization increased the ELISA titer. The mean ELISA titers from serum samples collected 4 weeks after each of the 4 immunizations in group 6 (Pv-Pv, $n = 4$) were 27,963, 579,689, 1,541,718, and 1,018,721, respectively. One-way ANOVA with post hoc analysis (Tukey) indicated that the $\log_{10}$-transformed ELISA titers 4 weeks after the first immunization were different from the titers 4 weeks after the subsequent 3 immunizations ($P < .0009$) and that the titers from 4 weeks after the second through fourth immunizations were not different ($P = .239$) from one another.

By comparing groups 2 and 6, one can evaluate the effect of a single protein immunization in animals that have and have not been primed with a DNA vaccine. In group 6, the mean ELISA titer 4 weeks after 1 protein immunization was 27,936, whereas the ELISA titer in group 2 after 3 DNA immunizations and 1 protein immunization was 567,490 ($P = .008$, Student's t test).

Another way to evaluate the priming with DNA is to compare it with priming with protein in adjuvant. After 3 doses of DNA or one dose of protein in adjuvant, antibody titers were 13,959 in monkeys primed with DNA and 27,936 in those primed with protein ($P = .02$, Student's t test). With an immunization with protein, antibody titers were increased 41-fold to 567,940 in the group primed with DNA and 20.7-fold to 579,689 in the group that received 2 doses of protein. Despite starting at a lower level, the monkeys primed 3 times with DNA and boosted with a single dose of protein achieved antibody titers equivalent to those for monkeys receiving 2 doses of protein.

As shown in table 1, we used ELISA, IFAT, and a blocking assay to evaluate pre- and postchallenge serum samples against recombinant protein and *P. falciparum*–infected erythrocytes and for blocking the binding of native *P. falciparum* EBA-175 to erythrocytes. We used simple linear regression to measure associations among these variables. Immediately before challenge (4 weeks after the fourth immunization), simple linear

Table 1. Titers for individual serum samples obtained 4 weeks after the fourth (boosting) immunization (prechallenge) and 4 weeks after infection with 10^4 *Plasmodium falciparum*–infected *Aotus* erythrocytes (postchallenge).

Animal	Prime boost	Prechallenge titer			Postchallenge titer		
		ELISA	IFAT	Blocking	ELISA	IFAT	Blocking
Group 1							
917	Dv-Dv	54,455	40,960	50,000	1,693,928	653,360	103,000
906	Dv-Dv	19,203	40,960	50,000	135,225	327,680	1,000,000
918	Dv-Dv	17,507	40,960	50,000	510,270	1,306,720	5,000,000
854	Dv-Dv	1761	10,240	50,000	16,937	163,840	5,000,000
Group 2							
913	**Dv-Pv**	**521,039**	**627,680**	**500,000**	**591,023**	**1,306,720**	**500,000**
919	**Dv-Pv**	**500,140**	**2,613,440**	**2,000,000**	**224,998**	**1,306,720**	**5,000,000**
909	Dv-Pv	952,841	627,680	5,000,000	675,440	2,613,440	5,000,000
898	**Dv-Pv**	**295,941**	**1,306,720**	**2,000,000**	**137,136**	**1,306,720**	**10,000,000**
Group 3							
899	Dv-DvPv	302,896	1,306,720	500,000	432,889	1,306,720	500,000
911	Dv-DvPv	287,251	627,680	500,000			1,400,000
908	Dv-DvPv	112,838	627,680	500,000	198,422	627,680	500,000
903	Dv-DvPv	210,201	1,306,720	200,000	122,890	1,306,720	
Group 4							
912	Dc-Dc	1592	80	0[a]	5315	81,920	100[a]
920	Dc-Dc	0	80		1173	81,920	
944	Dc-Dc	0	2560		0	81,920	
943	Dc-Dc	0	40		0	81,920	
Group 5							
948	Dc-DcPc	0	40	0[a]	0	81,920	6600[a]
950	Dc-DcPc	0	40		0	40,960	
954	Dc-DcPc	0	20		0	40,960	
931	Dc-DcPc	0	20		2721	40,960	
Group 6							
957	Pv-Pv	910,417	1,306,720	5,000,000	663,713	653,360	5,600,000
939	Pv-Pv	2,228,781	2,613,440	500,000	1,173,932	653,360	387,500
927	Pv-Pv	342,092	1,306,720	5,000,000	167,756	327,680	2,000,000
925	Pv-Pv	593,594	1,306,720	1,500,000	1,540,622	2,613,440	2,000,000
Group 7							
916	Pc-Pc	0	40	100[a]	0	40,960	0[a]
933	Pc-Pc	0	20		0	81,920	
921	Pc-Pc	0	20		0	20,480	
937	Pc-Pc	0	40		0	20,480	
Group 8							
935	n-n	0	40	0[a]	0	81,920	0[a]
938	n-n	0	1280		0	20,480	
946	n-n	0	20		0	81,920	
949	n-n	0	40		0	10,240	

NOTE. ELISA was performed using recombinant EBA-175, the 175-kD *P. falciparum* erythrocyte-binding protein, as the target antigen. *P. falciparum*–infected *Aotus* erythrocytes were used as the target in the immunofluorescent antibody test (IFAT). The blocking titer was obtained by preincubating [^{35}S]-metabolically labeled EBA-175 with serum samples. The titer of the serum able to block 50% of the binding of the labeled EBA-175 to normal human erythrocytes was interpolated. Protected, immunized monkeys are in bold. Dc, DNA vaccine control; Dv, *P. falciparum* EBA DNA vaccine; Pc, adjuvant control; n, nothing; Pv, *P. falciparum* EBA protein vaccine.

[a] Single value obtained from a pooled sample.

regression of ELISA and IFAT titers indicated that they explained 59% of each other's variance ($r^2 = .59$; $P < .0009$). The association between ELISA and blocking titers and between IFAT and blocking were less close ($r^2 = .22$, $P = .006$; and $r^2 = .25$, $P = .003$, respectively). After challenge, the associations were as follows: ELISA and IFAT ($r^2 = .39$; $P < .0009$); ELISA and blocking titer ($r^2 = .025$; $P = .41$); and IFAT and blocking titer ($r^2 = .29$; $P = .002$).

Infection with *P. falciparum* boosted the antibody titers in group 1, as measured by IFAT and blocking assay ($P = .003$ and .013, respectively; Student's t test). Antibody titers in groups 2, 3, and 6, which were much higher than those for group 1 before challenge, were not significantly boosted by infection.

IgG isolated from serum samples collected from the monkeys were tested in a growth inhibition assay to evaluate their ability to inhibit the growth of both FVO (sialic acid dependent) and 3D7 (sialic acid independent) strains of *P. falciparum* in vitro. Table 2 shows that monkeys receiving protein for the fourth immunization (groups 2, 3, and 6) had the highest levels of

Table 2. Mean percentage of inhibition (in reference to appropriate control groups) of growth or invasion by IgG isolated from serum samples obtained from 4 groups of *Aotus* monkeys 2 weeks after their final (fourth) immunization with various regimens of *Plasmodium falciparum* region II DNA and protein.

EBA-175 vaccine, group[a]	Control vaccine, group[a]	Percentage of inhibition[b] Experiment 1, FVO	Experiment 2 FVO	Experiment 2 3D7
Dv-Dv, 1	Dc-Dc, 4	52 (29)	42 (24)	20 (11)
Dv-Pv, 2	Dc-DcPc, 5	74 (49)	71 (46)	38 (21)
Dv-DvPv, 3	Dc-DcPc, 5	73 (42)	66 (42)	37 (21)
Pv-Pv, 6	Pc-Pc, 7	76 (51)	75 (50)	40 (23)

NOTE. IgG were pooled within each group and were tested at 1 mg/mL in 2-cycle growth inhibition assays against both the FVO (*Aotus* adapted) and 3D7 (human) strains of *P. falciparum*. Values represent mean percentage inhibition over 2 cycles in triplicate cultures by IgG from monkeys receiving EBA-175, the 175-kD *P. falciparum* erythrocyte-binding protein. The differences between experimental and control groups were statistically different in each case ($P < .05$, 2-tailed Student's t test). Final control parasitemias were 1.8% and 4% (FVO experiments 1 and 2, respectively) and 5% (3D7), representing control growth rates of ~7-, 10-, and 16-fold per cycle. Dc, DNA vaccine control; Dv, *P. falciparum* EBA DNA vaccine; Pc, adjuvant control; Pv, *P. falciparum* EBA protein vaccine.

[a] First letters of vaccine identify immunogen used in first 3 immunizations, second entry identifies immunogen(s) used in fourth immunization.

[b] Values in parentheses represent inhibitions per cycle as estimated from the percentage of inhibitions for the complete 2-cycle growth or invasion inhibition assay.

inhibitory activity relative to their appropriate control. These 3 groups were also significantly less likely to require treatment for elevated parasitemia than the groups not receiving protein for the fourth boost ($P = .034$, Fisher's exact test).

Response to challenge. We first analyzed whether there was a difference in peak parasitemias between all the control monkeys and all the monkeys immunized with EBA-175 in any regimen. The mean day of maximum parasitemia achieved in the monkeys was day 11.1 (range, 9–14 days). The geometric mean peak parasitemia in the 16 control animals was 410,133 parasites/μL, and, in the 16 animals that received EBA-175 vaccine, it was 194,161 parasites/μL ($P = .016$, Student's t test on $\log_{10}$-transformed values). We evaluated the relationship between receipt of EBA-175 (yes vs. no) and the distribution of peak parasitemia levels by quartile (figure 1). There was a statistically significant association between immunization with EBA-175 and having a lower peak parasitemia ($P = .029$, χ^2 test for trend). Of interest, the boost with a combination of DNA and recombinant protein in adjuvant (Dv-DvPv) seemed to adversely effect outcome on parasitemia, as it did with antibody responses. Peak parasitemias were significantly lower in the Dv-Pv group than they were in the Dv-DvPv group ($P = .0433$, Mann-Whitney U test).

Course of parasitemia. The course of parasitemia for each of the animals, separated by group, is presented in figure 2. None of the monkeys was parasitemic on days 1–3 after challenge; however, all became parasitemic on day 4, when the mean parasitemia density was 31 parasites/μL (95% confidence interval [CI], 23.3–38.9). We did not find a difference in the mean parasitemias on day 4 for the 16 monkeys infected in the first set, compared with the 16 infected in the second set ($P = .75$, Student's t test), which indicates that the 2 challenge subsets were comparable. Although there were no differences in mean parasitemia among the groups on day 4, we evaluated whether any of the groups diverged by day 9, the last treatment-free data collection day. (One animal in group 7 [Pc-Pc] and 1 in group 8 [n-n, naive controls] were treated for high parasitemia on day 9.) By day 9, groups 2 (Dv-Pv) and 6 (Pv-Pv) had lower mean parasitemias than did group 7 (Pc-Pc; $P = .015$, 1-way ANOVA with Tukey's HSD post hoc test). Other groups were not different from one another.

Threshold for treatment. After challenge, 5 of the 32 monkeys were protected, as defined by their successful control of parasitemia (i.e., they did not meet either of the 2 thresholds for drug treatment: $\geq$300,000 parasites/μL blood or hematocrit level >50% less than their prechallenge baseline level). Of the 27 that were treated, 21 had high parasitemia, and 6 were treated for low hematocrit levels (table 3). The mean day of treatment for monkeys being treated was day 11.9 (95% CI, 10.3–13.3). The mean day for treatment for elevated parasitemia was day 10.9 (95% CI, 10.5–11.4), whereas the mean day of treatment for low hematocrit was day 16.7 (95% CI, 12.8–20.5). The mean day of treatment for high parasitemia was different from the mean day of treatment for hematocrit ($P = .012$, Student's t test).

Among monkeys that reached the treatment threshold, a higher percentage of controls (93%, 13/14) than EBA-175–im-

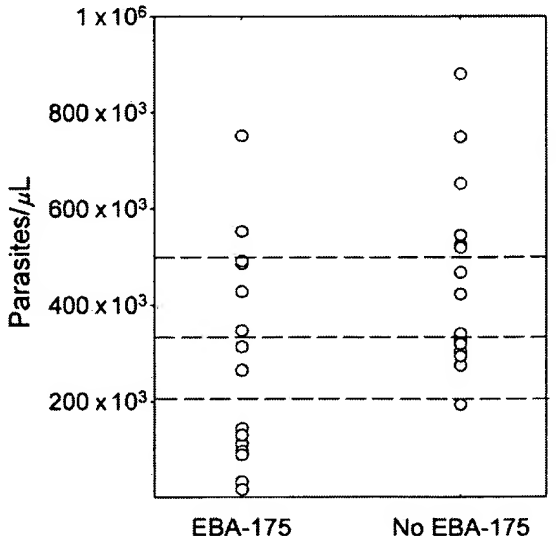

Figure 1. Maximum *Plasmodium falciparum* parasitemia achieved by each *Aotus* monkey. Animals are divided into 2 groups, depending on whether they were or were not immunized with the 175-kD *P. falciparum* erythrocyte-binding protein (EBA-175). Horizontal lines divide the data into quartiles.

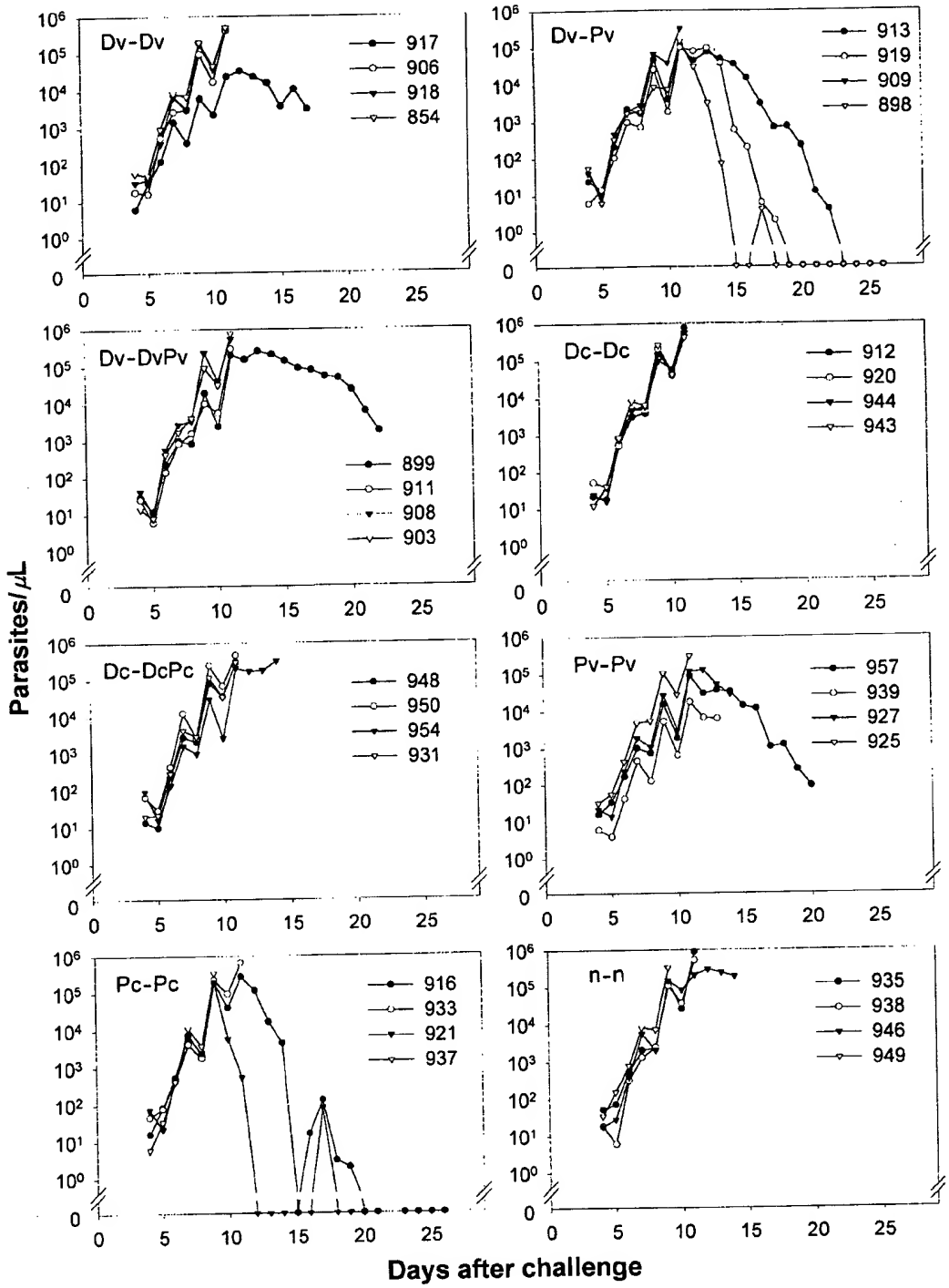

Figure 2. Parasitemia curves in *Aotus* monkeys challenged intravenously with 10^4 *Plasmodium falciparum*–infected *Aotus* erythrocytes. Results of all 8 study groups (4 monkeys/group) are shown. Dc, DNA vaccine control; Dv, *P. falciparum* EBA (erythrocyte-binding) DNA vaccine; n, nothing; Pv, *P. falciparum* EBA protein vaccine.

Table 3. Maximum parasitemia, minimum hematocrit (HTC) level, and treatment for 32 *Aotus* monkeys among 8 groups immunized with various regimens of *Plasmodium falciparum* EBA-175 region II DNA and protein and then challenged with 10^4 *P. falciparum*–infected erythrocytes.

Animal	Prime boost	Maximum no. parasites/μL, day	Minimum HTC %, day	Reason for treatment, day
Group 1				
917	Dv-Dv	33,861, 12	18, 17	Low HTC, 17
906	Dv-Dv	427,855, 11	38, 13	Parasitemia, 11
918	Dv-Dv	485,395, 11	32, 16	Parasitemia, 11
854	Dv-Dv	491,060, 11	30, 16	Parasitemia, 11
Group 2				
913	Dv-Pv	112,405, 11	27, 21	
919	Dv-Pv	96,366, 11	29, 16	
909	Dv-Pv	313,603, 11	44, 12	Parasitemia, 11
898	Dv-Pv	143,594, 11	40, 6	
Group 3				
899	Dv-DvPv	265,039, 13	16, 26	Low HTC, 22
911	Dv-DvPv	346,701, 13	23, 16	Parasitemia, 11
908	Dv-DvPv	552,737, 11	29, 16	Parasitemia, 11
903	Dv-DvPv	752,470, 11	23, 17	Parasitemia, 11
Group 4				
912	Dc-Dc	749,924, 11	32, 15	Parasitemia, 11
920	Dc-Dc	524,221, 11	21, 14	Parasitemia, 11
944	Dc-Dc	544,589, 11	30, 26	Parasitemia, 11
943	Dc-Dc	422,129, 11	21, 15	Parasitemia, 11
Group 5				
948	Dc-DcPc	300,428, 11	48, 6	Parasitemia, 11
950	Dc-DcPc	467,318, 11	26, 13	Parasitemia, 11
954	Dc-DcPc	328,370, 14	21, 15	Parasitemia, 14
931	Dc-DcPc	304,438, 11	20, 17	Parasitemia, 11
Group 6				
957	Pv-Pv	89,428, 11	19, 20	Low HTC, 20
939	Pv-Pv	17,335, 11	23, 13	Low HTC, 13
927	Pv-Pv	130,355, 12	21, 14	Low HTC, 14
925	Pv-Pv	313,561, 11	36, 21	Parasitemia, 11
Group 7				
916	Pc-Pc	275,095, 11	43, 23	
933	Pc-Pc	652,158, 11	43, 15	Parasitemia, 11
921	Pc-Pc	193,369, 9	44, 13	
937	Pc-Pc	340,336, 9	38, 12	Parasitemia, 9
Group 8				
935	n-n	880,279, 11	42, 14	Parasitemia, 11
938	n-n	518,365, 11	32, 16	Parasitemia, 11
946	n-n	293,936, 12	18, 16	Low HTC, 14
949	n-n	319,077, 9	39, 14	Parasitemia, 9
Mean		36,5181, 11.1	30.2, 15.8	

NOTE. A monkey was treated if it developed a parasitemia ≥300,000 parasites/μL or a >50% decrease in hematocrit level from the preinfection baseline level during the 28-day follow-up. Dc, DNA vaccine control; Dv, *P. falciparum* EBA (erythrocyte-binding) DNA vaccine; n, nothing; Pc, adjuvant control; Pv, *P. falciparum* EBA protein vaccine.

munized animals (62%, 8/13) developed parasitemias that exceeded the threshold for treatment (i.e., 300,000 parasites/μL blood; $P = .077$, Fisher's exact test). Among all the monkeys, the differences were similar (13/16 controls vs. 8/16 EBA-175 immunized; $P = .079$, χ^2 test). Of interest, a higher percentage of EBA-175–immunized than control monkeys reached the hematocrit threshold (5/16 vs. 1/16, respectively; $P = .085$, Fisher's exact test). Group 2 (Dv-Pv) had the highest percentage of protected animals (75%). The best control for group 2 was group 5 (Dc-DcPc). Comparison of protection levels in these 2 groups of 4 monkeys (75% vs. 0%) yielded a $P = .14$ (Fisher's exact test). Monkeys in the group that received 4 doses of EBA-175 protein in adjuvant, group 6 (Pv-Pv), controlled their parasitemia as well as, if not better than, the monkeys in group 2 (Dv-Pv) did, but 3 of the 4 had to be treated for decreased hematocrit levels (table 3).

Anemia. Six of the animals required treatment for decreased hematocrit levels (>50% decrease from prechallenge baseline level; table 3); 5 of the 6 were immunized with an EBA-175 vaccine, and all 5 were apparently controlling their parasitemias when they developed anemia (table 3). Among the EBA-175–immunized animals treated for decreased hematocrit ($n = 5$), the geometric mean peak parasitemia was 71,072, compared with 115,863 in the EBA-175–immunized animals that received no treatment at all ($n = 3$; $P = .48$, Student's t test).

We also used simple linear regression to look for an association between an individual animal's maximum parasitemia and its minimum hematocrit level. No association, positive or negative, was found ($r^2 = .048$).

The minimum hematocrit levels in animals receiving EBA-175 in either or both forms (DNA vaccine or recombinant protein vaccine, $n = 16$; mean minimum hematocrit level, 28%) were compared with those of animals not receiving EBA-175 ($n = 16$; mean minimum hematocrit, 32.4%). No association between minimum hematocrit level and receipt of EBA-175 vaccine was found ($P = .194$, Student's t test; power to resolve a true difference of 4.4 was 24%, whereas a true difference of 9.5 would yield a power of 80%).

A striking difference in the development of anemia was found between the Dv-Pv and Pv-Pv groups. Compared with the Dv-Pv group, the Pv-Pv group had slightly higher ELISA levels of antibodies after the booster vaccine (geometric mean [at an estimated OD of 0.5], 520,651 vs. 801,189; $P = .35$, Student's t test) and slightly lower peak parasitemias (166,492 vs. 137,670 parasites/μL; $P = .73$, Student's t test). One animal in each group reached the treatment threshold for parasitemia (i.e., 300,000 parasites/μL blood), but 3 of the 4 animals in the Pv-Pv group and none in the Dv-Pv group developed a >50% reduction in hematocrit level. Therefore, among the monkeys that did not reach the parasitemia threshold, the minimum hematocrit levels in the 3 monkeys in the Pv-Pv group (19%, 21%, and 23%, respectively; mean 21%) were significantly lower ($P = .037$, Student's t test on $\log_{10}$-transformed values) than those in the Dv-Pv group (27%, 29%, and 40%, respectively; mean 32%).

Discussion

These studies were designed to determine whether immunization of monkeys with *P. falciparum* EBA-175 region II vaccines leads to an antiparasite effect in vivo and in vitro and to compare DNA immunization, recombinant protein in adjuvant immunization, and prime-boost strategies for immunization. These results demonstrate for the first time that EBA-175 region

II immunization has an antiparasite effect in vivo and also confirm earlier studies demonstrating the induction of antiparasite antibodies with biological activity in vitro and demonstrate significant differences among immunization regimens in regard to induction of antibodies and protective immunity in vivo.

This study demonstrated a significant antiparasite effect in animals immunized with EBA-175 region II. The geometric mean peak parasitemia in monkeys that received EBA-175 region II, as DNA plasmid or as recombinant protein or a protein, was less than half that seen in monkeys not receiving EBA-175 region II in any form. Furthermore, the 7 lowest parasitemias were in the EBA-175–immunized animals, and 6 of the 8 highest parasitemias were in the control animals (figure 1).

The antiparasitic activity found in vivo in the monkeys is particularly encouraging because it is not likely that EBA-175 plays as significant a role in *P. falciparum* invasion of *Aotus* erythrocytes as we believe it does in the invasion of human erythrocytes. *Aotus* species are not natural hosts for *P. falciparum*. If EBA-175 binds to *Aotus* erythrocytes, it is clear that it does not bind as well to them as it does to human erythrocytes [30]. The genes encoding the orthologues of human glycophorins in *Aotus* have not been identified or characterized. The sialic acid types of *Aotus* species are also unknown. EBA-175 binds specifically to the α–2-3–linked sialic acid residues of O-linked tetrasaccharides on glycophorin A, whereas the α–2-6 linkages have lower binding affinities for EBA-175 [31]. Thus, the primary mechanism of invasion of *P. falciparum* merozoites into *Aotus* erythrocytes may not involve EBA-175. If EBA-175 does not play a critical role in *P. falciparum* merozoite invasion of *Aotus* erythrocytes, then the antiparasite activity that we have documented is almost certainly a reflection of recognition of surface-accessible EBA-175 on merozoites and resultant inhibition of invasion. Thus, there is reason to believe that the antiparasite activity in humans of anti–EBA-175 region II antibodies will be greater than that which we have documented in *Aotus* monkeys, because EBA-175 plays a crucial role in invasion of erythrocytes in humans.

Immunization with EBA-175 also induced IgG with significant growth inhibitory activity against the FVO strain of *P. falciparum* (table 2). Groups boosted with EBA-175 region II protein (groups 2, 3 and 6) had IgG with greater growth inhibitory activity than that for group 1, which was boosted with DNA. Significantly fewer animals in groups 2, 3, and 6 required treatment for elevated parasitemia. The growth of FVO was consistently inhibited more by the IgG than was the growth of 3D7. This may be due to the faster growth rate of the 3D7 strain, compared with the FVO strain in culture. Also, the sequences of the region II EBA-175 vaccines, both DNA and protein, used in this study are based on the FVO strain, but the 3D7 strain used in these experiments differs by only 1 residue [7]. Whether this single residue difference is the cause for the difference in inhibition between the 2 strains is unknown.

Another explanation is that, because 3D7 parasites can invade neuraminidase-treated erythrocytes [28], they partially escape the effects of anti–EBA-175 antibodies by using a different pathway for invasion of erythrocytes. Twelve of 15 *P. falciparum* field isolates in one study could invade neuraminidase-treated erythrocytes devoid of sialic acid residues [32]. We have shown, however, that antibodies against region II can still effectively inhibit merozoite invasion of 3D7 parasites, a strain that can invade neuraminidase-treated erythrocytes [28]. Antibody may be neutralizing free merozoites by binding to EBA-175 on their surfaces before they begin the attachment and invasion process. The fact that EBA-175 has such a common role in erythrocyte invasion clearly supports its inclusion in a multivalent malaria vaccine.

Three immunizations with a DNA vaccine followed by a single immunization with a recombinant protein vaccine (Dv-Pv) induced antibody levels equivalent to those induced by 4 recombinant protein immunizations (Pv-Pv). Both of these formulations produced antibody titers significantly higher than those seen in the group that received only DNA (Dv-Dv). The group that received 3 DNA vaccine immunizations and then both DNA and the recombinant protein for the fourth immunization (Dv-DvPv) had a mean antibody level less than half that of group 2 (Dv-Pv) but was not statistically different from either group 2 (Dv-Pv) or 6 (Pv-Pv). These data clearly illustrate the power of DNA priming to enhance the effect of a single protein immunization. After receiving a single protein immunization, the monkeys in the Pv-Pv group had a mean ELISA titer of 27,936, compared with a mean of 567,490 in the monkeys in group 2 after they received 3 DNA priming immunizations, followed by a single protein immunization. This is a 20.3-fold increase in titer attributable to DNA priming.

In this study, protection was defined as the ability of a given animal to eliminate its parasitemia without drug intervention. There were 2 different events that triggered treatment; a parasitemia ≥300,000 parasites/μL (7.5% parasitemia, assuming 4×10^6 erythrocytes/μL) or a >50% decrease in hematocrit level from prestudy baseline level. These triggers were selected because they protect the well-being of the animals while still providing a fair test of a blood-stage vaccine's efficacy.

In this study, 5 animals did not require treatment. Of the 5, 3 were in group 2, which received 3 immunizations with DNA vaccine and a single protein immunization. Group 5 was the most appropriate control group for group 2, and all 4 monkeys in group 5 had to be treated. Two monkeys in the adjuvant control group (group 7) did not require treatment. This, of course, made evaluation of protection in group 6 (Pv-Pv) difficult and raised the question of the mechanism of this nonspecific protection. Work in mice (authors' unpublished data) shows that a CpG ODN alone can protect 50%–100% of mice receiving a *Plasmodium yoelii* sporozoite challenge. This type of protection differs from that in the Pc-Pc group (group 7) in a few important ways. In the mouse study, the mice received

the CpG ODN no more than 7 days before challenge with sporozoites, whereas, in this study, the monkeys received their last dose of CpG ODN 30 days before challenge with blood-stage parasites. Also complicating this picture is the observation that group 6 (Pv-Pv) and group 7 (Pc-Pc) received the same CpG ODN regimen, and, although none of the monkeys in group 6 was protected, only 1 required treatment for elevated parasitemia. It is also interesting that 1 dose of adjuvant containing CpG ODN (group 5) did not appear to have this effect.

There was an association between receiving EBA-175 recombinant protein as the final (fourth) immunization and not reaching the threshold for treatment of elevated parasitemia ($\geq$300,000 parasites/μL). Only 5 (42%) of 12 monkeys receiving the EBA-175 recombinant protein as the final dose required treatment for elevated parasitemia, whereas 16 (80%) of 20 not receiving the EBA-175 protein as the fourth dose required treatment ($P = .034$, Fisher's exact test).

There was a difference in the day of treatment, depending on whether treatment was administered for parasitemia or anemia. Treatment for low hematocrit levels was started, on average, 6 days later than treatment for elevated parasitemia. Some of the episodes of declining hematocrit level in these animals were quite dramatic, so we searched for factors that may be associated with the onset of this anemia. We did not find an association between the maximum parasitemia and the subsequent development of anemia, nor did we find any association between receiving EBA-175 region II in either form (DNA vaccine or recombinant protein) and anemia. However, the sample sizes were small, and it is interesting to note that, among the 3 group 6 (Pv-Pv) and 3 group 2 (Dv-Pv) monkeys that were controlling their parasitemias, all 3 in the Pv-Pv group developed anemia requiring treatment, whereas none of the 3 in the Dv-Pv group required treatment. These reductions of >50% in hematocrit levels in monkeys with parasitemias of <5% generally occurred over only a few days, indicating rapid removal of nonparasitized erythrocytes. Of interest, we have found a similar reduction in hematocrit levels in *Aotus* monkeys immunized with *P. falciparum*–infected erythrocytes [33], and others have observed a similar anemia in *Aotus vociferans* immunized with merozoite surface protein–1 [34]. We are currently evaluating the pathogenesis of the anemia, and preliminary findings do not support an antibody-mediated mechanism for it (data not shown).

There have now been a number of studies reporting protection of *Aotus* monkeys against challenge with *P. falciparum*–infected erythrocytes either by immunization with native protein [35] or with subunit vaccines [36, 37]. In all cases, induction of protection required Freund's complete adjuvant. The group sizes in our studies ($n = 4$) were relatively small and did not allow for the demonstration of statistically significant protection between group 2 (Dv-Pv) and its control group (group 5) and must be repeated. Nonetheless, our study showing protection of *Aotus* monkeys without Freund's complete adjuvant provides an impetus for further evaluation of the prime-boost strategy and of the adjuvant combination (Montanide plus CpG ODN) [27]. It also provides a potential replacement for Freund's complete adjuvant in monkey studies with a combination adjuvant. Both the CpG and Montanide components have been evaluated independently in humans and have been shown to increase the magnitude of the antibody response and to be well tolerated (A. M. Krieg, personal communication) [38, 39].

To our knowledge, this study demonstrated for the first time in vivo that immunization with EBA-175 region II induced an immune response having a significant antiparasitic effect, particularly in its ability to limit the maximum parasitemia observed after blood-stage challenge. In addition, we have demonstrated that a prime-boost strategy using 3 DNA vaccine priming immunizations followed by a protein boost produced antibody levels equivalent to those of 4 protein immunizations. These limited data also suggested that this heterologous immunization, prime-boost strategy produced higher levels of protection against challenge than did a homologous immunization strategy.

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